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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, C12P 19/34
G01N 33/48, 33/566, C07H 15/12

A1

(11) International Publication Number: WO 91/15601

(43) International Publication Date: 17 October 1991 (17.10.91)

(21) International Application Number: PCT/US91/02211

(22) International Filing Date: 4 April 1991 (04.04.91)

(30) Priority data:
504,591 5 April 1990 (05.04.90) US
Not furnished 15 March 1991 (15.03.91) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: MODIFIED RNA TEMPLATE-SPECIFIC POLYMERASE CHAIN REACTION

(57) Abstract

The present invention relates to methods of detecting an RNA sequence by tagging the sequence with a unique random nucleotide sequence during reverse transcription. The unique nucleotide sequence is then utilized to selectively amplify the resulting DNA sequence. The present invention reduces the number of false positives obtained as a result of contaminating DNA.

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# MODIFIED RNA TEMPLATE-SPECIFIC POLYMERASE CHAIN REACTION

The present application is a continuation-in-part application of Serial No. 07/504,591 filed April 5, 1990, which is hereby incorporated in its entirety.

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

The present invention relates to a method for detecting an RNA sequence. More specifically, the present invention relates to a method of amplifying an RNA sequence using a modification of the polymerase chain reaction.

### 2. Background Information

The polymerase chain reaction (PCR) method, developed by Perkin-Elmer-Cetus Corporation, is a popular and extraordinarily powerful technique for the amplification of DNA sequences. It has wide-ranging applications including molecular biology, medical diagnostics, genetics, forensics, and archeology [Saiki et al., Science 230, 1350 (1985); Saiki et al., Science 239, 487 (1985); Kogan et al., N. Engl. J. Med. 316, 656 (1987); Higuchi et al., Nature 332, 543 (1988); and Paabo et al., J. Biol. Chem. 264, 9709 (1989)]. When coupled with reverse transcription (RT-PCR), this technique can detect as few as 1 to 100 copies of a specific RNA from single cells or small numbers of cells. [Kawasaki et al., Proc. Natl. Acad. Sci. U.S.A. 85, 5698 (1988); Rappolee et al., Science 241, 708 (1988); Rappolee et al., Science 241, 1823 (1988); Sarkar et al., Science 244, 331 (1988); and Frohman et al., Proc. Natl. Acad. Sci. U.S.A. 85, 8998 (1988)].

Unfortunately, the exquisite sensitivity of this technique presents one of its severe shortcomings, false positives resulting from contamination with minute quantities of DNA [Kwok et al., Nature 339, 237 (1989); Sakar et al., Nature 343, 27 (1989)]. Potential sources of contaminating DNA may include: 1) endogenous sources such as small quantities of genomic DNA which may copurify with RNA, and 2) exogenous sources such as cDNA, plasmid DNA, or DNA fragments amplified in previous PCRs (i.e. carryover). While the frequency of false positives can be reduced somewhat by instituting and maintaining special techniques (e.g. premixing and aliquoting reagents; use of disposable gloves and positive displacement pipettes; and adding the experimental sample last), contamination still remains a major problem, especially when low abundance RNA transcripts are being sought [Kwok et al., Nature 339, 237 (1989); Lo, Y.-M., et al., Lancet 2, 699 (1988)].

Conventional RT-PCR amplifies equally well DNA derived from an RNA template or from a DNA template. Therefore, small quantities of contaminating DNA from virtually any source may easily result in false positives. Assuming approximately 4 pg of genomic DNA per mammalian haploid cell, and a sensitivity of 1 to 100 copies, conventional RT-PCR would result in false positives from only picogram quantities of contaminating genomic DNA.

It is possible to avoid false positives caused by amplification of genomic DNA which may copurify with RNA if the target sequence to be amplified by RT-PCR spans an intron. However, this experimental design is

not always possible since 1) some genes do not contain introns in convenient regions, and 2) the genomic structure of many target genes are not yet known.

In the laboratory of the present inventors, RT-PCR was recently used to detect small quantities of Xenopus insulin mRNAs in unfertilized eggs and early embryos. Despite the fact that numerous precautions were taken to exclude contamination of Xenopus insulin cDNAs which had been previously cloned in our laboratory [Shuldiner et al., J. Biol. Chem. 264,9428 (1989)], frequent false positives precluded meaningful interpretation of the experiments.

The present invention, RNA template-specific PCR (RS-PCR) and modified RS-PCR, provides methods of detecting minute quantities of RNA without the problems of false positives associated with RT-PCR. In the present methods the reduction in the frequency of false positives is achieved without sacrificing sensitivity obtained with conventional RT-PCR.

## SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method of detecting an RNA sequence which reduces the number of false positives resulting from DNA contamination in the sample (i.e., previously cloned cDNAs, genomic DNA or carryover of DNA amplified in previous PCRs). The present method increases the accuracy of the procedure without sacrificing sensitivity.

It is another object of the present invention to provide a method of detecting an RNA sequence which obviates the necessity to choose a target RNA sequence which spans an intron.

Various other objects and advantages of the present invention will be apparent from the following description of the invention and the drawings.

In one embodiment, the present invention relates to a method of detecting an RNA sequence. The method comprises:

i) reverse transcribing the RNA sequence from an oligonucleotide primer hybridized thereto which oligonucleotide primer ($d_{17}$-$t_{30}$) comprises:

a) on the 3' end thereof (segment $d_{17}$), a nucleotide sequence complementary to a region of the RNA sequence to be detected; and

b) on the 5' end thereof (segment $t_{30}$), a unique random nucleotide sequence or tag whereby a single stranded DNA sequence is produced which has at its 5' end the unique sequence;

ii) hybridizing, at a temperature high enough to preclude annealing of the $d_{17}$ segment of the $d_{17}$-$t_{30}$ primer to possible contaminating DNA, but low enough to allow annealing, an upstream oligonucleotide primer ($u_{30}$), to a region of said DNA sequence to which it is complementary, a predetermined distance upstream from $t_{30}$;

iii) extending the primer ($u_{30}$) so that a DNA molecule is produced having at its 3' end a nucleotide sequence complementary to said unique nucleotide sequence (segment $t_{30}$);

iv) denaturing the double-stranded DNA molecule produced in step (iii);

v) hybridizing, at a temperature high enough to preclude annealing of the $d_{17}$ segment of the $d_{17}$-$t_{30}$ primer to possible contaminating DNA, but low enough to

allow annealing the upstream PCR oligonucleotide primer ($u_{30}$) to the region of said DNA sequence to which it is complementary and,

hybridizing, at a temperature high enough to preclude annealing of the $d_{17}$ segment of the $d_{17}$-$t_{30}$ primer to possible contaminating DNA, but low enough to allow annealing of a PCR oligonucleotide primer ($t_{30}$) comprising all or a portion of said unique nucleotide sequence, to the 3' end of said DNA sequence to which it is complementary;

vi) extending the primers ($u_{30}$) and ($t_{30}$) thereby producing two DNA molecules; and

vii) detecting the presence or absence of the amplified DNA sequence;

wherein the $d_{17}$ segment of the oligonucleotide primer $d_{17}$-$t_{30}$ does not hybridize to contaminating DNA at the annealing temperature of the PCR, and oligonucleotide primer $u_{30}$ and oligonucleotide primer $t_{30}$ do hybridize to their appropriate DNA templates at the annealing temperature of the PCR.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows diagrammatically the RNA template-specific PCR method (RS-PCR).

FIGURE 2 compares the sensitivity of conventional reverse transcriptase - PCR (RT-PCR) and novel RS-PCR when beginning with an RNA template.

FIGURE 3 compares conventional RT-PCR and novel RS-PCR when DNA rather than RNA is used as a starting template to mimic DNA contamination.

FIGURE 4 shows the effect of changing the nucleotide sequence of the unique segment of oligonucleotide primer $d_{20}$-$t_{21}$.

FIGURE 5 shows schematically the modified RNA template-specific PCR.

FIGURE 6 compares conventional RT-PCR and modified RS-PCR. RT primer $d_{17t}30$: PCR primers $t_{30}$ and $u_{30}$ (lanes 1-4); RT primer $d_{30}t$: PCR primers $d_{30}$ and $u_{30}$ (lanes 6-9). Lane 0 is a HaeIII digest of PhiX174 DNA, while lane 5 is RS-PCR in the absence of any template.

FIGURE 7 shows PCR carryover contamination is ignored with modified RS-PCR. Lanes 1 and 3; RT primer $d_{17}$-$t_{30}$, PCR primers $u_{30}$ and $t_{30}$. Lanes 2 and 4; RT primer $d_{16}t'_{30}$, PCR primers $u_{30}$ and $t'_{30}$.

FIGURE 8 shows the region of Xenopus insulin RNA that was used as the target RNA to test the modified RS-PCR procedure. Reverse transcription primer $d_{17}$-$t_{30}$ contained a 17 base sequence at its 3' end (segment $d_{17}$) that was complementary (antisense) to a region of Xenopus insulin RNA in the 3' untranslated region (nucleotides 404-420), and 30 bases at its 5' end (segment $t_{30}$) that were unique in sequence. Upstream primer $u_{30}$ is identical (sense) to Xenopus insulin RNA in the coding region (nucleotides 59-88).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention, RNA template-specific PCR (RS-PCR) and modified RS-PCR, relates to a targeted amplification method which distinguishes RNA in the sample from contaminating DNA and amplifies only sequences derived from RNA. Minute quantities of cDNA, plasmid DNA or carryover DNA amplified in previous PCRs can be important sources of contamination when using conventional RT-PCR. The present invention reduces the number of false positives obtained as a result of contaminating DNA. Furthermore, the present invention

obviates the necessity of choosing a target RNA sequence which spans an intron in order to distinguish the reverse transcribed DNA from contaminating genomic DNA. In addition, the modified RS-PCR eliminates the need for removal of the primer after reverse transcription, such as by ultrafiltration.

The RS-PCR method of the present invention is shown schematically in Figure 1.

In the first step, a first oligonucleotide primer designated $d_{20}$-$t_{21}$ in Figure 1 (advantageously, of about 41 nucleotides) is hybridized to the RNA sequence to be detected. Primer $d_{20}$-$t_{21}$ comprises on the 3' end, a nucleotide sequence (advantageously, about 20 nucleotides) complementary to the 3' end of the RNA sequence whose presence is to be detected (segment $d_{20}$), and on the 5' end, a unique random nucleotide sequence or tag (advantageously, about 21 nucleotides) (segment $t_{21}$). While the 3' end of the primer hybridizes to the RNA sequence, the 5' end of the primer remains unhybridized as no complementary sequence exists within the sample.

Once primer $d_{20}$-$t_{21}$ has been hybridized to the 3' end of the RNA sequence, reverse transcriptase is used to extend the primer. The resulting single -(-) stranded DNA segment is thus tagged at its 5' end with the unique sequence $t_{21}$ of original primer $d_{20}$-$t_{21}$. This unique 5' sequence ($t_{21}$) distinguishes between DNA generated from the RNA-template and possible contaminating DNA.

It is preferable for the unique sequence to be composed of approximately equal amounts of each nucleotide (i.e. about 25% of each nucleotide).

Furthermore, it is preferable to choose a unique sequence which is unlikely to have significant secondary structure, and does not contain significant complementarity at its 3' end with the 3' end of the upstream primer (for example, primer $u_{21}$). The sequence can also be selected so as to contain a convenient restriction enzyme recognition site if desired. One skilled in the art can easily generate by computer appropriate sequences, 5'-GACAAGCTTCAGGTAATCGAT-3' and 5'-CCGAATTCTGTAGTCCGTCA-3' being two examples.

Prior to amplification, excess primer $d_{20}$-$t_{21}$ is removed by ultrafiltration through a Centricon 100 device (Amicon, Danvers, MA) or similar device.

In the second and third steps of the present method, the DNA segment resulting from the previous step is amplified using the PCR technique (see U.S. Patents 4,683,202 and 4,683,195). Two oligonucleotide primers designated $u_{21}$ and $t_{21}$ in Figure 1 are utilized to amplify the DNA. Upstream oligonucleotide primer $u_{21}$ (advantageously, about 21 nucleotides) comprises a nucleotide sequence complementary the single stranded DNA segment produced in Step 1, a predetermined distance upstream from primer $d_{20}t_{21}$. Oligonucleotide primer $t_{21}$ (advantageously, about 21 nucleotides) comprises the unique nucleotide sequence with which the segment of DNA was tagged during reverse transcription. The two primers are added to the sample and the PCR is carried out.

In the second step of the present method (PCR cycle 1), primer $u_{21}$, which is complementary to a region of the single stranded DNA segment produced in Step 1, a predetermined distance upstream from primer

$d_{20}t_{21}$, hybridizes thereto and is extended therefrom creating a complementary strand of DNA which includes at its 3' end a sequence complementary to the unique sequence. Primer $t_{21}$ is not utilized in the first PCR cycle since no complementary sequence is present in the sample. However, primer $t_{21}$ is used in the second PCR cycle and all cycles thereafter.

In the third step of the present method, the double stranded DNA segment resulting from the first PCR cycle, is denatured prior to the second PCR cycle.

For the second cycle and all subsequent PCR cycles, primer $u_{21}$ which is complementary to the 3' end of the single - (-) stranded DNA hybridizes thereto and is extended therefrom. At the same time, the primer $t_{21}$ hybridizes to its complementary sequence at the 3' end of the single - (+) strand of DNA and is extended therefrom. All DNA synthesis occurs in the 5' to 3' direction.

The modified RS-PCR method of the present invention is shown schematically in Figure 5.

The modified RS-PCR method eliminates the need to remove the first oligonucleotide primer, designated $d_{17}$-$t_{30}$ in Figure 5, by selecting oligonucleotide primers $d_{17}$-$t_{30}$, $t_{30}$ and $d_{30}$, so that differential hydridization occurs under the PCR conditions. The primers are selected so that the $d_{17}$-$t_{30}$ primer and the $d_{30}$ and the $t_{30}$ primers anneal under different temperatures.

In the first step, as with the RS-PCR method, a first oligonucleotide primer designated $d_{17}$-$t_{30}$ in Figure 5 (advantageously, of about 47 nucleotides) is hybridized to the RNA sequence to be detected. Primer $d_{17}$-$t_{30}$ comprises on the 3' end, a nucleotide sequence

(advantageously, about 17 nucleotides) complementary to the 3' end of the RNA sequence whose presence is to be detected (segment $d_{17}$), and on the 5' end, a unique random nucleotide sequence or tag (advantageously, about 30 nucleotides) (segment $t_{30}$).

The primer should be selected so that the length of the d segment is such that it will not anneal efficiently to any DNA contaminants at the elevated annealing temperatures used in Steps 2 and 3. One skilled in the art can easily generate by computer suitable $d_{17}$-$t_{30}$ primers including, for example, 5'-gaacatcgatgacaagcttaggtatcgatatgatggaattgccttga-3' and 5'-cttatacggatatcctggcaattcggacttgcatgatggaattgcc-3'.

Once primer $d_{17}$-$t_{30}$ has been hybridized to the RNA sequence, reverse transcriptase is used to extend the primer thereby creating a single -(-) stranded DNA segment which is tagged at its 5' end with the unique sequence, the $t_{30}$ segment, of original primer. This unique 5' sequence, as with the RS-PCR method, distinguishes between DNA generated from the RNA-template and possible contaminating DNA.

In the second step of the present method (PCR cycle 1), oligonucleotide primer designated $u_{30}$ in Figure 5 (advantageously, about 30 nucleotides) hybridizes to the single stranded DNA generated in Step 1 a predetermined distance upstream from primer $d_{17}$-$t_{30}$, and is extended therefrom creating a complementary strand of DNA which includes at its 3' end a sequence complementary to the unique sequence. The primer $u_{30}$ comprises a nucleotide sequence complementary to the single stranded DNA segment produced in Step 1, a predetermined distance upstream from primer $d_{17}t_{30}$. The

annealing stage of the PCR cycle is carried out at a temperature high enough to preclude annealing of the $d_{17}$ segment of the reverse transcription primer $d_{17}$-$t_{30}$ a to contaminating DNA, but low enough to allow annealing of PCR primer $u_{30}$, for example temperatures of 42° C or greater.

In the third step of the present method, the double stranded DNA segment resulting from the first PCR cycle, is denatured prior to the second PCR cycle.

For the second cycle and all subsequent cycles, primer $u_{30}$ which is complementary to the single - (-) stranded DNA hybridizes thereto and is extended therefrom. At the same time, a second oligonucleotide primer, designated $t_{30}$ in Figure 5 (advantageously, about 30 nucleotides) is added to the sample. The primer $t_{30}$ comprises the unique nucleotide sequence with which the segment of DNA was tagged during reverse transcription. When the primer is added to the sample it hybridizes to its complementary sequence at the 3' end of the single - (+) strand of DNA and is extended therefrom. The annealing stage of all PCR cycles is conducted at a temperature high enough to preclude annealing of the $d_{17}$ segment of the reverse transcription primer $d_{17}$-$t_{30}$ a to contaminating DNA, but low enough to allow annealing of PCR primers $u_{30}$ and $t_{30}$.

With RS-PCR, sequences derived from RNA that are tagged with the unique sequence ($t_{30}$) during reverse transcription (step 1) are amplified preferentially during PCR (steps 2 and 3). The original RS-PCR method requires ultrafiltration after reverse transcription to remove excess RT primer [Shuldiner et al., Gene 91, 139

(1990)]. The modified RS-PCR cirumvents this step by increasing the length of the RT and PCR primers, and increasing the PCR annealing temperature. The primers are selected so that the RT primer, $d_{17}$-$t_{30}$, hybridizes to the RNA template under the reverse transcription conditions but does not hybridize to possible DNA contaminants under the PCR conditions.

The longer length of the $u_{30}$ and $t_{30}$ primers allows annealing to occur at an increased temperature, that is temperatures up to about 72° C. Annealing of the 17 base $d_{17}$ segment of the RT primer $d_{17}$-$t_{30}$ occurs efficiently during reverse transcription at 37°C, but not at the higher PCR annealing temperature. Thus, when Steps 2 and 3 are carried out at a temperature of 42° C or greater (preferably 65° or greater), remaining $d_{17}$-$t_{30}$ primer does not anneal to possible DNA contaminants while the $u_{30}$ and $t_{30}$ primers will anneal and be extended.

In both RS-PCR methods described above, each cycle of PCR involves primer hybridization, extension to yield double stranded DNA and denaturation. After the first PCR cycle, both the (+) and (-) strands of the DNA serve as templates from which a new strand of DNA is created. This leads to logarithmic expansion of the tagged segment of DNA.

Contaminating DNA lacks the unique nucleotide sequence. Thus, during the PCR the 3' end of the single - (-) strand of DNA serves as a template for primer $u_{21}$ or $u_{30}$ (but the 3' end of the -(+) strand can not act as template for unique primer $t_{21}$ or $t_{30}$ since there is no complementary sequence). This allows only linear amplification which, as one skilled in the art

knows, does not produce enough DNA to result in a false positive when detecting the presence of the logarithmically amplified PCR product.

Potential contamination arising from carryover of PCR products from previous experiments in which a different unique sequence was used is virtually eliminated when the present invention is used. With the methods of the present invention, no false positives were observed in over 20 independent experiments. The criteria for selecting the unique sequence of the primer used for reverse transcription and subsequent PCR is that the sequence selected is not present in the sample i.e. is unique. Therefore, the sequence used can be changed periodically. Changing the unique sequence prevents amplification of carryover PCR products. Thus, the methods of the present invention are particularly useful in a clinical laboratory setting where many samples and automation make careful laboratory hygiene more difficult.

The present invention is as sensitive as the well known PCR and RT-PCR procedures. Therefore, the small quantity of RNA needed is not affected. However, the present invention has the advantage of being more accurate.

In the following non-limiting examples, a segment of Xenopus insulin RNA is amplified by the present methods. The methods are applicable to the amplification of other RNAs.

## Examples

### RS-PCR

Example 1: RNA Template-Specific Polymerase Chain Reaction (RS-PCR)

Xenopus insulin mRNA was amplified using novel RS-PCR, which involves first reverse transcribing Xenopus pancreatic RNA using an oligonucleotide 41-mer as a primer (oligonucleotide $d_{20}$-$t_{21}$) whose nucleotide sequence contained 20-bases at the 3'-end which were complementary to a region of Xenopus insulin mRNA (segment $d_{20}$), and 21-bases at the 5'-end which consisted of a unique random sequence selected by computer or similar method (segment $t_{21}$) (FIGURE 1) followed by PCR amplification of the DNA segment.

As a first step, total RNA from Xenopus pancreatic tissue was prepared by the guanidinium isothiocyanate method [Chirgwin et al., Biochemistry 18, 5294 (1979)]. RNA was reverse transcribed at 42°C for one hour in a 25 µl reaction mixture containing Tris-HCl (50 mM, pH adjusted to 8.7 at room temperature), NaCl (100 mM), $MgCl_2$ (6 mM), dithiothreitol (10 mM), dNTP's (1 mM each), RNasin (1 µl; Promega Biotec; Madison, WI), oligonucleotide $d_{20}$-$t_{21}$ (5'-GACAAGCTTCAGGTATCGATTTGCATGATGGAATTGCCTTG-3'; 0.5 µM), and AMV-reverse transcriptase (10 units; Promega Biotec).

This reverse transcription step resulted in single-(-) stranded DNA which had been "tagged" at its 5' end with a unique 21-nucleotide sequence or tag (segment $t_{21}$).

After reverse transcription, the oligonucleotide primer $d_{20}$-$t_{21}$ was efficiently removed (>99.9%) using a Centricon 100 ultrafiltration device (Amicon; Danvers, MA) according to manufacturer's recommendations. Then PCR was performed using as primers oligonucleotide $t_{21}$, a 21-mer containing the

same unique nucleotide sequence as in segment $t_{21}$ of oligonucleotide $d_{20}$-$t_{21}$ and oligonucleotide $u_{21}$, a 21-mer complementary to the first strand, 244 bp upstream from oligonucleotide $t_{21}$.

PCR amplification was performed in a 50 µl reaction volume containing Tris-HCl (10 mM, pH adjusted to 8.3 at room temperature), KCl (50 mM), $MgCl_2$ (1.5 mM), gelatin (0.01%), dNTP's (200 µM each), oligonucleotide $t_{21}$ (5'-GACAAGCTTCAGGTAATCGAT-3'; 0.5, µM), oligonucleotide $u_{21}$ (5'-GAGGCTTCTTCTACTACCCTA-3'; 0.5 µm) and Taq polymerase (1 units; Perkin Elmer-Cetus Corp., Emeryville, CA). The reaction mixture was covered with paraffin oil (approximately 50 µl), heated to 94°C for 5 minutes, followed by PCR (45-60 cycles). Each cycle consisted of annealing (55°C, 1.5 min), extension (72°C, 1.5 min) and denaturation (94°C, 1 min) except for the last cycle, in which the extension time was increased to 15 minutes to insure completeness of extension.

Twenty microliters of the reaction mixtures were loaded onto a composite gel consisting of 1% agarose and 2% Nusieve GTG (FMC Bioproducts; Rockland, ME) in Tris-borate-EDTA buffer, electrophoresed, stained with ethidium bromide, and visualized by UV transillumination.

Since logarithmic amplification is dependent upon nucleotide sequences corresponding to $d_{20}$, $t_{21}$ and $u_{21}$, only sequences derived from Xenopus insulin RNA which had been reverse transcribed with oligonucleotide $d_{20}$-$t_{21}$ were amplified logarithmically, and contaminating DNA, which lacks the oligonucleotide $t_{21}$ sequence was not amplified logarithmically.

Example 2: Comparison of novel RS-PCR and conventional RT-PCR using an RNA template.

To test whether novel RS-PCR was as sensitive as conventional RT-PCR, Xenopus pancreatic RNA which had been reverse transcribed with oligonucleotide $d_{20}$-$t_{21}$ and ultrafiltered, was subjected to either conventional RT-PCR (oligonucleotides $d_{20}$ and $u_{21}$), or novel RS-PCR (oligonucleotides $t_{21}$ and $u_{21}$). PCR with either of these two oligonucleotide pairs resulted in similar sensitivity (FIGURE 2).

Xenopus pancreatic RNA (1 ng) was reverse transcribed and ultrafiltered according to the methods of Example 1.

For the conventional RT-PCR, 60 cycles of the PCR were performed on serial ten-fold dilutions of reverse transcribed and ultrafiltered pancreatic RNA with oligonucleotide primers $d_{20}$ and $u_{21}$ (FIGURE 2, lanes 1-5) using the conditions described in Example 1.

For the novel RS-PCR comparison, identical serial dilutions of the reverse transcribed and ultrafiltered pancreatic RNA was amplified by PCR using oligonucleotide primers $t_{21}$ and $u_{21}$ (FIGURE 2, lanes 6-9).

The predicted 244-bp and 265-bp amplified bands observed on the ethidium bromide-stained gel hybridized strongly to a radiolabeled full-length Xenopus insulin cDNA probe [Southern J. Mol. Biol. 98, 503 (1975)].

PCR with either the oligonucleotide pair $d_{20}$ and $u_{21}$ or the pair $t_{21}$ and $u_{21}$ resulted in similar sensitivity. Conventional RT-PCR with or without

removal of excess oligonucleotide $d_{20}$-$t_{21}$ by Centricon 100 ultrafiltration resulted in similar sensitivity, as did reverse transcription with oligonucleotide $d_{20}$ as the primer rather than oligonucleotide $d_{20}$-$t_{21}$. These results suggest that neither Centricon 100 ultrafiltration or reverse transcription using an oligonucleotide with a random 21-nucleotide overhang at its 5' end result in a significant decrease in sensitivity.

Example 3: Comparison of novel RS-PCR and conventional RT-PCR using a DNA template

By contrast to the Example 2 where the sensitivity of the reaction was not affected by the use of the unique nucleotide sequence, novel RS-PCR was approximately 10 to 1000-fold less affected by the presence of DNA contaminants (i.e., Xenopus insulin cDNA) than conventional RT-PCR even after 60 cycles (FIGURE 3).

Full-length Xenopus insulin cDNA (300 pg) was "reverse transcribed" with oligonucleotide $d_{20}$-$t_{21}$, excess oligonucleotide $d_{20}$-$t_{21}$ removed by ultrafiltration, and PCR (60 cycles) was accomplished as described in the above Examples. Results of the conventional RT-PCR performed on serial ten-fold dilutions of the "reverse transcribed" and ultrafiltered Xenopus insulin cDNA using oligonucleotides $d_{20}$ and $u_{21}$ is shown in FIGURE 3, lanes 1-5. Novel RS-PCR of identical serial ten-fold dilutions of "reverse transcribed" and ultrafiltered Xenopus insulin cDNA using oligonucleotides $t_{21}$ and $u_{21}$ is shown in lanes 6-10 of the same figure.

In theory, with the RS-PCR method, only RNA that had been primed with oligonucleotide $d_{20}$-$t_{21}$ during RT should have been amplified during PCR. However, it was found that when relatively large quantities of DNA template (>10 pg or approximately 1 x $10^7$ molecules) were used, detectable amplification was observed (lane 6 in Fig. 3). It has been determined from separate experiments that this phenomenon was caused by two mechanisms; i) at relatively high DNA concentrations, RT acted as a DNA polymerase and incorporated oligonucleotide $d_{20}$-$t_{21}$ into the so-called first strand, and ii) the minute quantities of oligonucleotide $d_{20}$-$t_{21}$ that remained behind after ultrafiltration incorporated into DNA during early PCR cycles which could then be amplified efficiently in RS-PCR.

Example 4: Effect of changing the sequence of the unique segment $t_{21}$ of oligonucleotide $d_{20}$-$t_{21}$ on conventional RT-PCR and novel RS-PCR.

In order to evaluate the ability of the RS-PCR method to eliminate problems of carryover contamination of amplified DNA from previous RS-PCR experiments which had been tagged with a different unique sequence $t_{21}$ the following experiment was conducted.

Xenopus pancreatic RNA (1 ng) was reversed transcribed with either oligonucleotide 41-mer $d_{20}$-$t_{21}$ (FIGURE 4, lanes 1, 2, 3 and 7), or oligonucleotide 41-mer $d_{20}$-$t'_{21}$ (5'-CCGAATTCTGTAGTCCGTCATTGCAGATGGAATTGCCTTG-3') (FIGURE 4, lanes 4-6). After ultrafiltration, PCR (45 cycles) was accomplished as described in the previous Examples using oligonucleotide pairs $t_{21}$ and $u_{21}$ (FIGURE 4, lanes

1 and 4), $t'_{21}$ (5'-CCGAATTCTGTAGTCCGTCA-3') and $u_{21}$ (FIGURE 4, lanes 2 and 5), $d_{20}$ and $u_{21}$ (FIGURE 4, lanes 3 and 6), or $t_{21}$ and $u'_{21}$ (5'-TGACCTTTCCAGCACTTATC-3') (FIGURE 4, lane 7).

As expected, the RNA that had been reversed transcribed with oligonucleotide $d_{20}$-$t_{21}$ was amplified only when oligonucleotide $t_{21}$ was used during PCR, but not when an unrelated unique 21-mer (oligonucleotide $t'_{21}$) was used. Conversely, reverse transcription of Xenopus pancreatic RNA with oligonucleotide $d_{20}$-$t'_{21}$, could only be amplified by the corresponding unique 21-mer, oligonucleotide $t'_{21}$, and not by the unrelated random 21-mer, oligonucleotide $t_{21}$. As expected, when conventional RT-PCR was used (i.e. oligonucleotide $d_{20}$), amplification occurred regardless of whether reverse transcription primers $d_{20}$-$t_{21}$ or $d_{20}$-$t'_{21}$ were used.

MODIFIED RS-PCR

Example 5: Modified RS-PCR

Oligonucleotides were synthesized on a Coder 300 automated DNA synthesizer (E.I. Du Pont Company; Wilmington, DE), and purified with NENsorb Prep columns (New England Nuclear; Boston, MA) according to the manufacturer's directions (see Table 1 below). Xenopus insulin (sense) RNA was prepared by ligating an 890 bp Xenopus insulin cDNA [Shuldiner et al., J. Biol. Chem. 264, 9428 (1989)] into pSP71 (Promega Biotec; Madison, WI).

After linearization of the recombinant plasmid with BglII, T7 RNA polymerase (Promega Biotec) was used for in vitro transcription to generate Xenopus insulin (sense) RNA. The RNA was purified by oligo-dT cellulose chromatography (Bethesda Research

Laboratories). Only full-length RNA was retained by the column since the 3' end contained a long poly-A tail. The RNA was quantitated by UV absorbance at 260 nm.

RNA was diluted to the appropriate concentration in water containing yeast tRNA (100 μg/ml) (Bethesda Research Laboratories). DNA templates used to demonstrate RNA specificity were either a double-stranded 890 bp Xenopus insulin cDNA insert [Shuldiner et al., J. Biol. Chem. 264, 9428 (1989)], or a 377 bp Xenopus insulin RS-PCR product that had been subjected to ultrafiltration with a Millipore-MC-100 device (Millipore; Bedford, MA) to remove excess primers. DNA templates were quantitated by comparison to a known quantity of a HaeIII digest of PhiX174 (Bethesda Research Laboratories) after agarose gel electrophoresis.

Table I. Primer sequences used to compare improved RS-PCR to conventional RT-PCR.

| Primer | Sequence |
|---|---|
| $d_{17}t_{30}$ | 5'-GAACATCGATGACAAGCTTAGGTATCGATATGATGGAATTGCCTTGA-3' |
| $t_{30}$ | 5'-GAACATCGATGACAAGCTTAGGTATCGATA-3' |
| $d_{16}t'_{30}$ | 5'-CTTATACGGATATCCTGGCAATTCGGACTTGCATGATGGAATTGCC-3' |
| $t_{30}$ | 5'-CTTATACGGATATCCTGGCAATTCGGACTT-3' |
| $d_{30}$ | 5'-GCATGATGGAATTGCCTTGAAGGTGCCTTG-3' |
| $u_{30}$ | 5'-ATGCAGTGTCTGCCCCTGGTTCTTGTCCTC-3' |

Reverse transcription of serial ten-fold dilutions of Xenopus insulin RNA ($10^7$ to $10^4$ copies) was accomplished at 37° C in a final volume of 20 µl containing KCl (50 mM), Tris-HCl (10 mM; pH 8.3 at 25° C), $MgCl_2$ (1.5 mM), gelatin (0.01 mg/ml), dNTPs (200 µM each), RNasin (40 U; Promega Biotec), AMV-reverse transcriptase (7 U; Promega Biotec), and primer $d_{17}$-$t_{30}$ (0.5 µM).

Primer $d_{17}$-$t_{30}$ (Table I) was a 47-mer whose sequence contained 17 bases at its 3'-end that were complementary to a region of Xenopus insulin mRNA, designated segment $d_{17}$, and 30 bases at its 5'-end that were unique in sequence, designated segment $t_{30}$. Thus, reverse transcription yields single-stranded DNA that contains a unique 30 base "tag" (segment $t_{30}$) at its 5' end (FIGURE 5).

The second strand was synthesized during the first cycle of PCR in which 5 µl of the RT reaction mixture from step 1 was used directly in a final volume of 50 µl containing KCl (50 mM), Tris-HCl (10 mM; pH 8.3 at 25° C), $MgCl_2$ (1.5 mM), gelatin (0.01 mg/ml), dNTPs (200 µM each), upstream primer $u_{30}$ (0.5 µM), downstream primer $t_{30}$ (0.5 µM) and Taq polymerase (1.5 U; Perkin Elmer-Cetus; Emeryville, CA).

Upstream (sense) primer $u_{30}$ was a 30-mer corresponding to Xenopus insulin cDNA that was 347 bp upstream from the sequence corresponding to segment $d_{17}$, while downstream primer $t_{30}$ was a 30-mer whose sequence was identical to segment $t_{30}$ of RT primer $d_{17}$-$t_{30}$ (see Table I). With these primers, sequences derived from RNA that had been tagged with unique

sequence ($t_{30}$) during reverse transcription were amplified logarithmically preferentially, while contaminating DNAs, lacking the unique tag, were not amplified logarithmically (FIGURE 5) [Shuldiner et al., Gene 91, 139 (1990)].

After covering the PCR reaction mixture with parafin oil (approximately 50 $\mu$l), 35 cycles of PCR were performed, each cycle consisting of denaturation (94° C, 1 min) and annealing/extension (70° C, 2 min). In the first cycle, the denaturation time was increased to 5 min, and in the last cycle, the annealing/extension time was increased to 10 min to ensure completeness of the extension.

Twenty microliters of the PCR reaction mixture was electrophoresed on a composite gel consisting of 1% agarose (Bethesda Research Laboratories) and 2% Nuseive GTG (FMC Bioproducts; Rockland, ME). DNA was visualized by ethidium bromide staining and UV transillumination.

Example 6: Comparison of modified RS-PCR and conventional RT-PCR

To compare the sensitivity of modified RS-PCR to conventional RT-PCR, serial ten-fold dilutions of Xenopus insulin RNA ($10^7$ to $10^4$ molecules) were amplified using either modified RS-PCR (RT primer $d_{17}$-$t_{30}$; PCR primers $u_{30}$ and $t_{30}$) (FIGURE 6, panel a, lanes 1-5), or conventional RT-PCR (RT primer $d_{30}$; PCR primers $u_{30}$ and $d_{30}$) (FIGURE 6, panel a, lanes 6-9).

Modified RS-PCR was equally sensitive to conventional RT-PCR when beginning with an RNA template. By contrast, when Xenopus insulin double-

stranded DNA ($10^7$ copies) was used as starting template to mimic DNA contamination, conventional RT-PCR resulted, as expected, in a strong signal (FIGURE 6, panel b, lane 11), while the modified RS-PCR method virtually ignored theDNA template (FIGURE 6, panel b, lane 10). When larger amounts of DNA were used (i.e., > $10^8$ copies), a faint signal was detected with RS-PCR [Shuldiner et al., Gene 91, 139 (1990)].

To mimic RS-PCR carryover contamination, RS-PCR was performed with two 377 bp Xenopus insulin RS-PCR products (approximately $10^8$ copies) that were identical to each other except each contained a different unique tag (sequence $t_{30}$ or $t'_{30}$ (Table I and FIGURE 7).

Double-stranded Xenopus insulin DNA containing either tag sequences $t_{30}$ (FIGURE 7, lanes 1 and 2), or $t'_{30}$ (FIGURE 7, lanes 3 and 4) were subjected to improved RS-PCR as described expect 30 cycles were performed. Amplification of each DNA template occurred efficiently when the primers matched the unique tag present in the PCR product (FIGURE 7 lanes 1 and 4). However, when RS-PCR primers were used that did not match the unique tag present in the PCR product, no amplification occurred (FIGURE 7, lanes 2 and 3). Thus, carryover contamination of RS-PCR products in which one unique tag was used is virtually eliminated when RS-PCR is performed with a different unique tag.

* * * * *

The entire contents of all publications cited hereinabove are hereby incorporated by

reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A method of detecting an RNA sequence comprising the steps of:

i) reverse transcribing the RNA sequence from an oligonucleotide primer hybridized thereto which oligonucleotide primer ($d_{20}$-$t_{21}$) comprises:

a) on the 3' end thereof, a nucleotide sequence complementary to the 3' end of the RNA sequence (segment $d_{20}$); and

b) on the 5' end thereof, a unique random nucleotide sequence or tag whereby a single stranded DNA sequence is produced which has at its 5' end said unique sequence (segment $t_{21}$);

ii) removing excess oligonucleotide primer ($d_{20}$-$t_{21}$);

iii) hybridizing an upstream oligonucleotide primer ($u_{21}$) complementary to the 3' end of said DNA sequence thereto;

iv) extending the primer ($u_{21}$) so that a DNA molecule is produced having at its 3' end a nucleotide sequence complementary to said unique nucleotide sequence;

v) denaturing the double-stranded DNA molecule produced in step (iv);

vi) hybridizing the oligonucleotide primer ($u_{21}$) to the 3' end of said DNA sequence to which it is complementary and hybridizing an oligonucleotide primer ($t_{21}$) comprising all or a portion of said unique nucleotide sequence, to the 3' end of said DNA sequence to which it is complementary;

vii) extending the primers ($u_{21}$) and ($t_{21}$) thereby producing two DNA molecules; and

viii) detecting the presence or absence of the amplified DNA sequence.

2. The method according to claim 1 further comprising before step (viii) repeating steps v-vii multiple times.

3. The method according to claim 1 wherein said oligonucleotide primer ($d_{20}$-$t_{21}$) is about 41 nucleotides in length.

4. The method according to claim 1 wherein said oligonucleotide primers ($u_{21}$) and ($t_{21}$) are each about 21 nucleotides in length.

5. The method according to claim 1 wherein said unique random nucleotide sequence is comprised of approximately 25% of each nucleotide.

6. The method according to claim 1 wherein said RNA sequence is a segment of mRNA.

7. The method according to claim 6 wherein said segment of mRNA is a segment of insulin mRNA.

8. The method according to claim 7 wherein said insulin mRNA is Xenopus insulin mRNA.

9. The method according to claim 1 wherein said unique nucleotide sequence is 5'-GACAAGCTTCAGGTAATCGAT-3'.

10. The method according to claim 1 wherein said unique nucleotide sequence is 5'-CCGAATTCTGTAGTCCGTCA-3'.

11. The method according to claim 1 wherein said segment of RNA is contaminated with DNA.

12. A method of detecting an RNA sequence comprising the steps of:

i) reverse transcribing the RNA sequence from an oligonucleotide primer hybridized thereto which oligonucleotide primer ($d_{17}$-$t_{30}$) comprises:

a) on the 3' end thereof (segment $d_{17}$), a nucleotide sequence complementary to a region of the RNA sequence; and

b) on the 5' end thereof (segment $t_{30}$), a unique nucleotide sequence or tag whereby a single stranded DNA sequence is produced which has at its 5' end said unique sequence;

ii) hybridizing an upstream oligonucleotide primer ($u_{30}$), complementary to said DNA sequence, to a region of said sequence, at a temperature selected so that said $d_{17}$ segment does not anneal to contaminating DNA but so that said primer $u_{30}$ does anneal;

iii) extending the primer ($u_{30}$) so that a DNA molecule is produced having at its 3' end a nucleotide sequence complementary to said unique nucleotide sequence (segment $t_{30}$);

iv) denaturing the double-stranded DNA molecule produced in step (iii);

v) hybridizing the oligonucleotide primer ($u_{30}$) to said DNA sequence to which it is complementary and hybridizing an oligonucleotide primer ($t_{30}$) comprising all or a portion of said unique nucleotide sequence, to a region of said DNA sequence to which it is complementary,

wherein said hybridization is carried out at a temperature selected so that said $d_{17}$ segment does not anneal to contaminating DNA but so that said primers $u_{30}$ and $t_{30}$ do anneal;

vi) extending the primers ($u_{30}$) and ($t_{30}$) thereby producing two DNA molecules; and

vii) detecting the presence or absence of the amplified DNA sequence;

13. The method according to claim 12 wherein said hybridization is carried out at a temperature 42° C or greater.

14. The method according to claim 12 further comprising before step (vii) repeating steps iv-vi multiple times.

15. The method according to claim 12 wherein said hybridization in steps ii and v occurs at a temperature between 65° and 72° C.

16. The method according to claim 12 wherein said oligonucleotide primer ($d_{17}$-$t_{30}$) is about 47 nucleotides in length.

17. The method according to claim 12 wherein said oligonucleotide primers ($u_{30}$) and ($t_{30}$) are each about 30 nucleotides in length.

18. The method according to claim 12 wherein said unique random nucleotide sequence is comprised of approximately 25% of each nucleotide.

19. The method according to claim 12 wherein said RNA sequence is a segment of mRNA.

20. The method according to claim 18 wherein said segment of mRNA is a segment of insulin mRNA.

21. The method according to claim 19 wherein said insulin mRNA is Xenopus insulin mRNA.

# RNA Template-Specific PCR (RS-PCR)

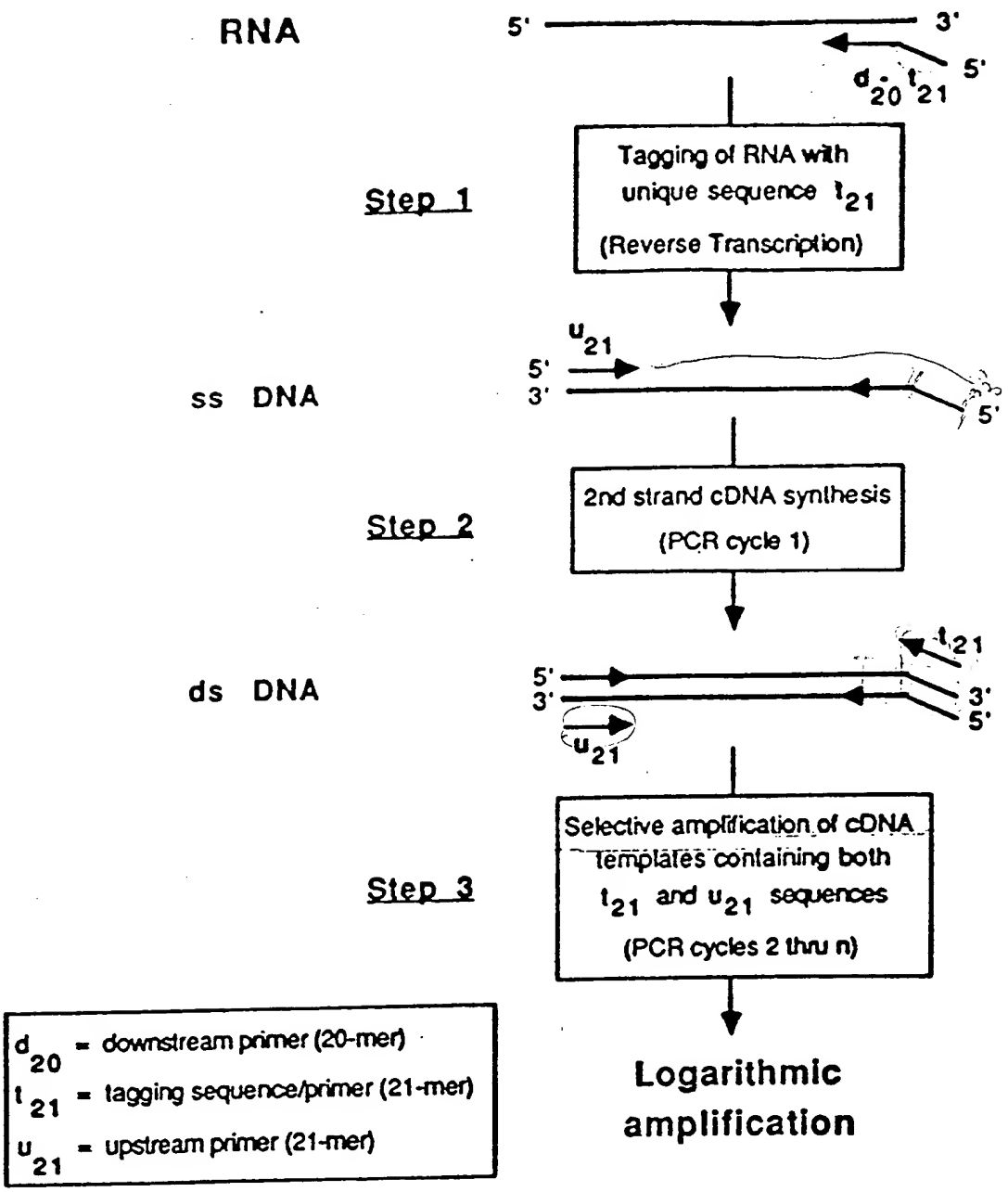



FIGURE 1

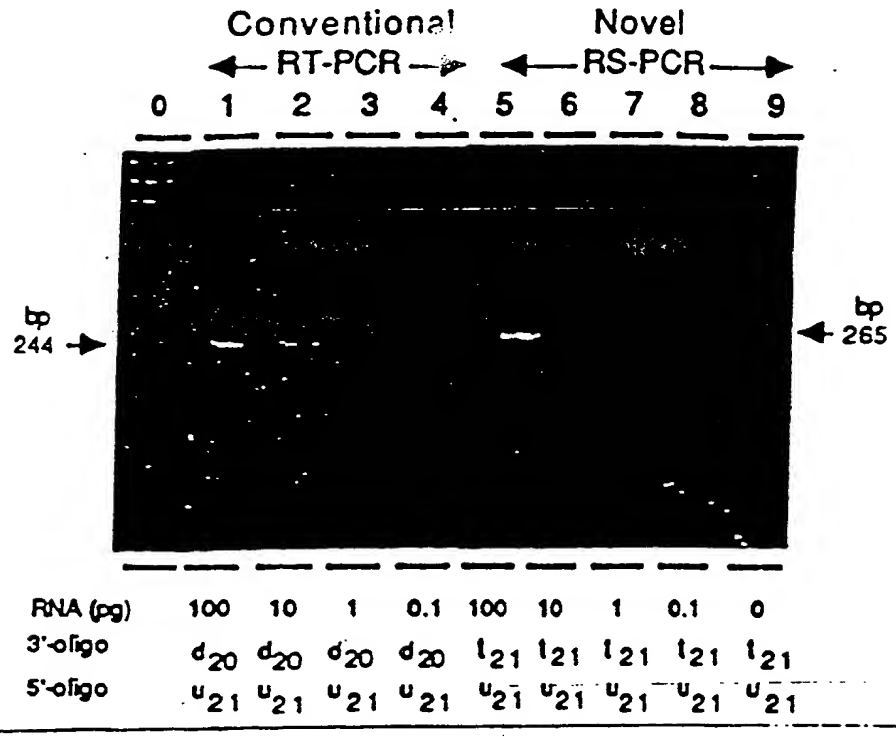

Conventional
RT-PCR
Novel
RS-PCR
0 1 2 3 4 5 6 7 8 9
bp 244
bp 265
RNA (pg) 100 10 1 0.1 100 10 1 0.1 0
3'-oligo d20 d20 d20 d20 t21 t21 t21 t21 t21
5'-oligo u21 u21 u21 u21 u21 u21 u21 u21 u21

FIGURE 2

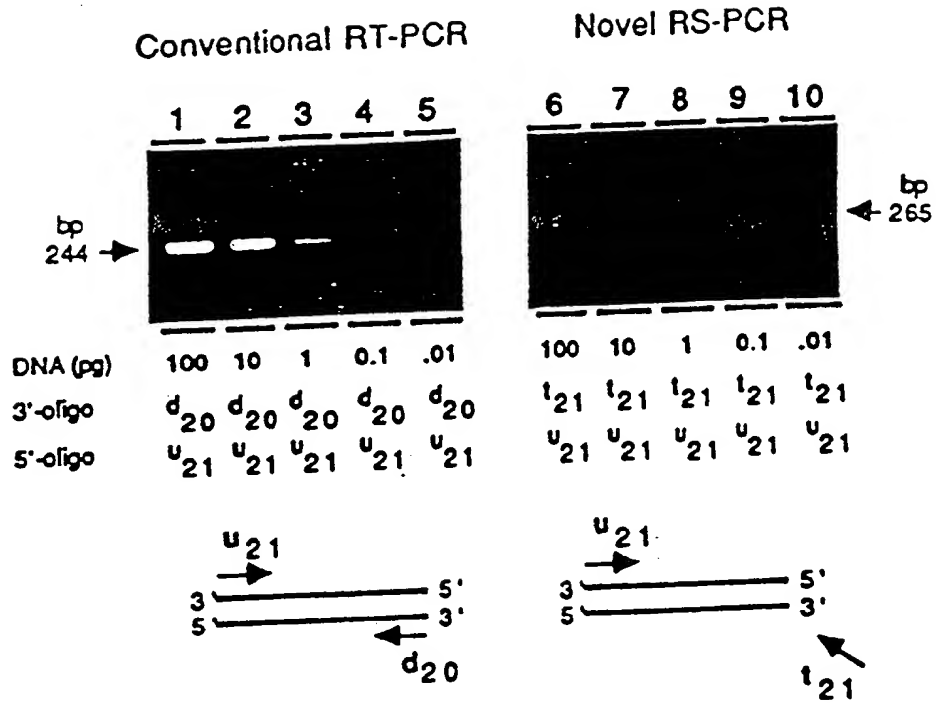
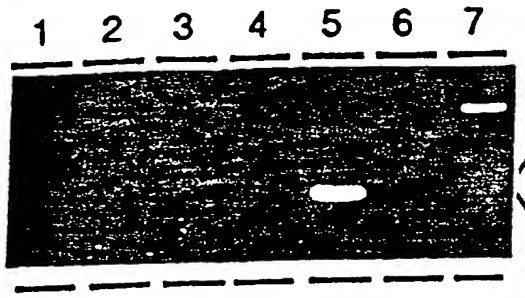

Conventional RT-PCR
Novel RS-PCR
1 2 3 4 5
6 7 8 9 10
bp
244
bp
265
DNA (pg)
100 10 1 0.1 .01
100 10 1 0.1 .01
3'-oligo
$d_{20}$ $d_{20}$ $d_{20}$ $d_{20}$ $d_{20}$
$t_{21}$ $t_{21}$ $t_{21}$ $t_{21}$ $t_{21}$
5'-oligo
$u_{21}$ $u_{21}$ $u_{21}$ $u_{21}$ $u_{21}$
$u_{21}$ $u_{21}$ $u_{21}$ $u_{21}$ $u_{21}$
$u_{21}$
3
5
5'
3'
$d_{20}$
$u_{21}$
3
5
5'
3'
$t_{21}$

FIGURE 3

RS-PCR: Effect of changing the unique random sequence




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| RT primer tag | $t_{21}$ | $t_{21}$ | $t_{21}$ | $t'_{21}$ | $t'_{21}$ | $t'_{21}$ | $t_{21}$ |
| 3'-PCR oligo | $t_{21}$ | $t'_{21}$ | $d_{20}$ | $t_{21}$ | $t'_{21}$ | $t'_{21}$ | $t_{21}$ |
| 5'-PCR oligo | $u_{21}$ | $u_{21}$ | $u_{21}$ | $u_{21}$ | $u_{21}$ | $u_{21}$ | $u'_{21}$ |
| RNase (mg/ml) | 0 | 0 | 0 | 0 | 0 | 1 | 0 |

FIGURE 4

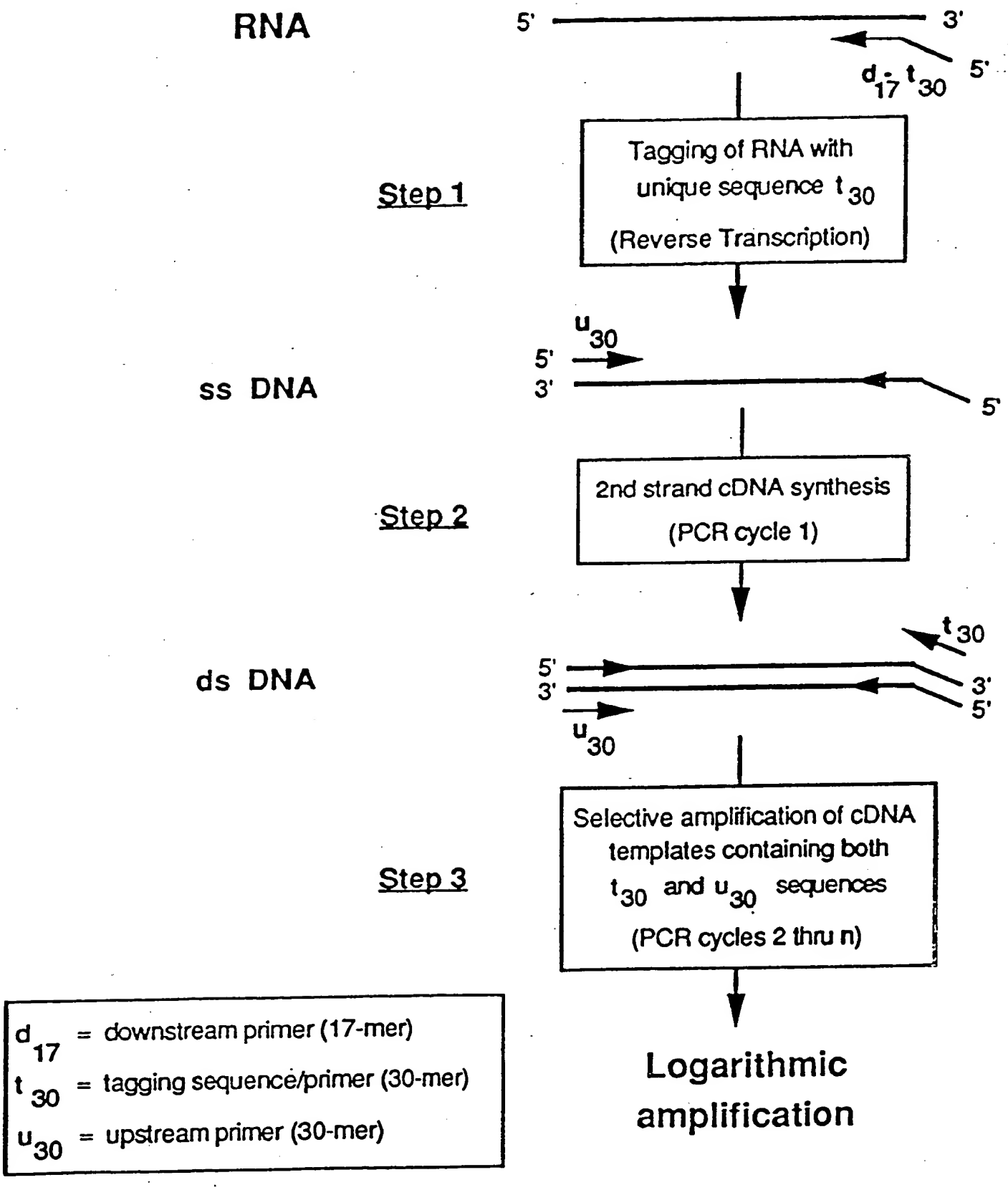

RNA
5'
3'
5'
$d_{17}$ $t_{30}$
Step 1
Tagging of RNA with unique sequence $t_{30}$
(Reverse Transcription)
$u_{30}$
5'
3'
5'
ss DNA
Step 2
2nd strand cDNA synthesis
(PCR cycle 1)
$t_{30}$
5'
3'
3'
5'
$u_{30}$
ds DNA
Step 3
Selective amplification of cDNA templates containing both $t_{30}$ and $u_{30}$ sequences
(PCR cycles 2 thru n)
Logarithmic amplification
$d_{17}$ = downstream primer (17-mer)
$t_{30}$ = tagging sequence/primer (30-mer)
$u_{30}$ = upstream primer (30-mer)

FIGURE 5

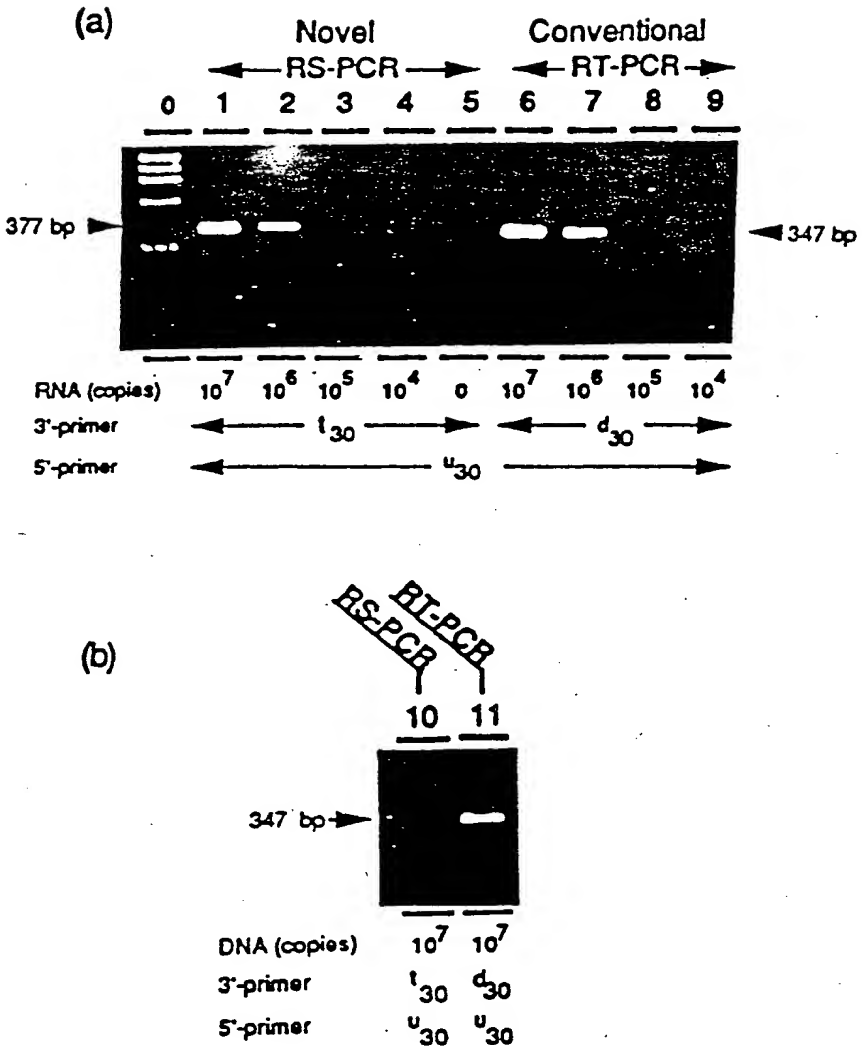

(a)
Novel
Conventional
RS-PCR
RT-PCR
0 1 2 3 4 5 6 7 8 9
377 bp
347 bp
RNA (copies) $10^7$ $10^6$ $10^5$ $10^4$ 0 $10^7$ $10^6$ $10^5$ $10^4$
3'-primer $t_{30}$ $d_{30}$
5'-primer $u_{30}$
(b)
RS-PCR
RT-PCR
10 11
347 bp
DNA (copies) $10^7$ $10^7$
3'-primer $t_{30}$ $d_{30}$
5'-primer $u_{30}$ $u_{30}$

FIGURE 6

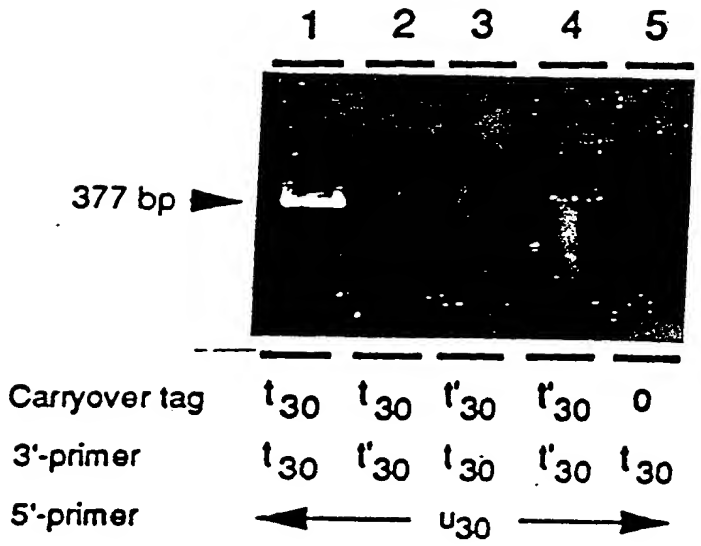

1
2
3
4
5
377 bp
Carryover tag
$t_{30}$ $t_{30}$ $t'_{30}$ $t'_{30}$ 0
3'-primer
$t_{30}$ $t'_{30}$ $t_{30}$ $t'_{30}$ $t_{30}$
5'-primer
$u_{30}$

FIGURE 7

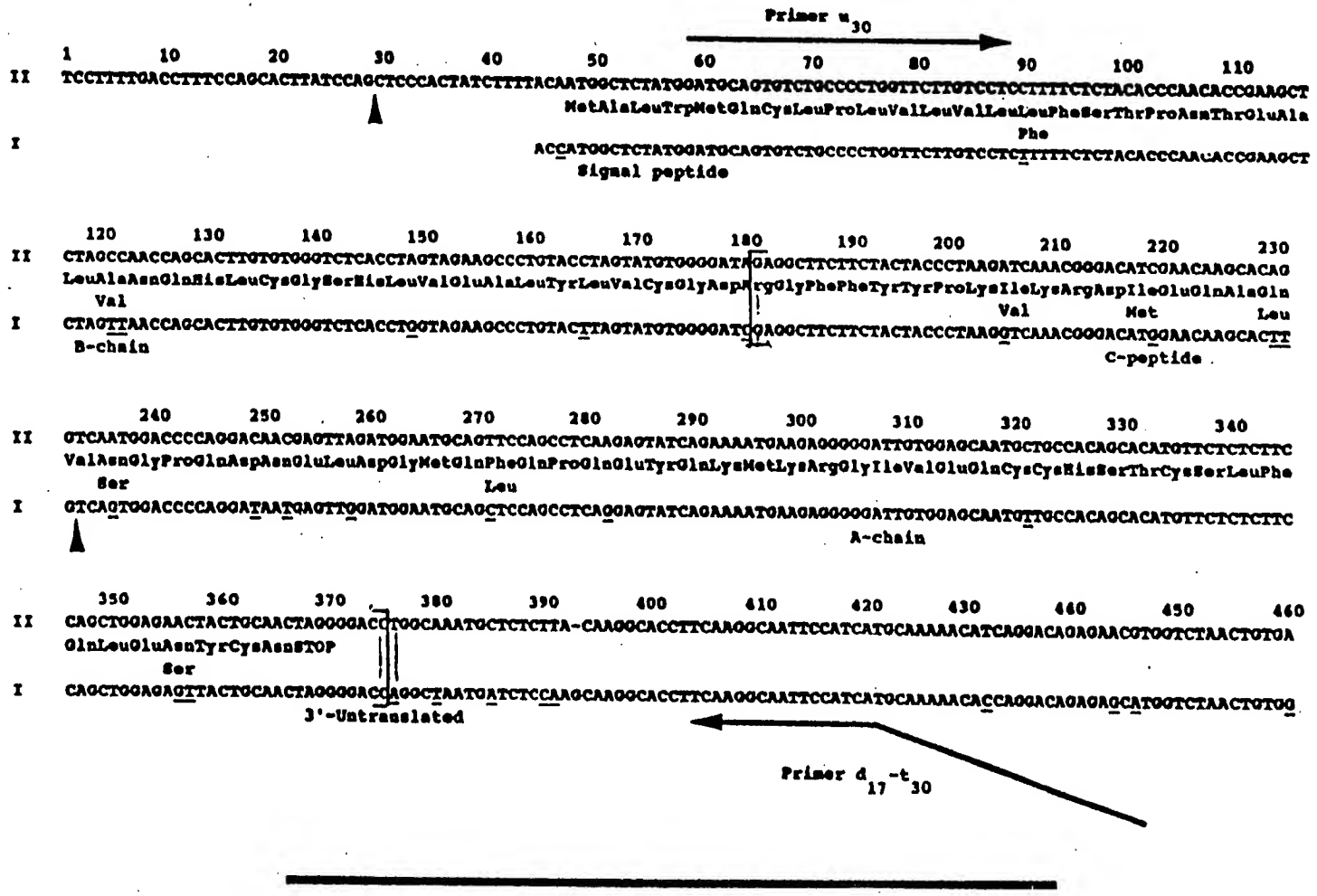

Primer u30
Signal peptide
B-chain
C-peptide
A-chain
3'-Untranslated
Primer d17-t30
Predicted RS-PCR Product - 377 bp
Internal Probe - 196 bp

FIGURE 8

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02211

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68; C12P 19/34; G01N 33/48, 33/566; C07H 15/12
US CL: 435/6, 91; 436/94, 501; 536/26, 27, 28, 29; 935/77, 78

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| USCL | 435/6, 91; 436/94, 501; 536/26, 27, 28, 29; 935/77, 78 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| Y | US, A, 4,683,195 (MULLIS et al) 28 July 1987, See column 6, lines 62-64, column 7, lines 66-68; column 10, lines 10-13; column 29, example 9. | 1-21 |
| Y | Science, Vol. 243, issued 13 January 1989, E. Y. LOH et al., "Polymerase Chain Reaction with Single-Sided Specificity: Analysis of T Cell Receptor Delta Chain", pages 217-220; see page 218, Figure 1. | 1-21 |

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 24 June 1991 | 05 AUG 1991 |

| International Searching Authority | Signature of Authorized Officer |
|---|---|
| ISA/US | Stephanie W. Zitomer<br>Stephanie W. Zitomer, Ph.D. |

Form PCT/ISA/210 (second sheet) (rev. 11-87)